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13. Abstract ***(Maximum 200 Words)*** Investigation of the molecular events underlying progression of prostate cancer to androgen-independence has been impeded by the lack of an in vivo model that yields "pure" populations of prostate cancer cells that are not contaminated with host cells. Here we characterize a new in vivo model that employs hollow fibers and allows for the retrieval of uncontaminated prostate cancer cells during various stages of endocrine progression to androgen-independence. Prostate-specific antigen (PSA) gene expression, proliferation of cells, and histology were examined before and after castration of the host. Approximately $5x10^6$ LNCaP prostate cancer cells/animal provided measurable levels of PSA in the serum that increased in intact (non-castrated) animals, decreased 80% to a nadir following castration, and subsequently increased by 4 weeks after castration indicating progression to androgen-independence. In vivo proliferation of LNCaP cells inside fibers continued in the presence of androgens and continued to increase, albeit at a slower rate, in castrated animals. Gene expression patterns in this model were consistent with clinical samples and other models when using cDNA arrays to screen RNA isolated from the model. Approximately 150 genes were identified to be altered during progression to androgen independence with 77 of these genes having unknown function.

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INTRODUCTION

Presently the only therapy for advanced prostate cancer is to reduce the patient's level of androgen. Unfortunately, androgen withdrawal does not completely or permanently eliminate all prostate cancer cells, a pre-requisite for a cure. Eventually the disease will return in an androgen-independent form. An early sign of progression to androgen independence in patients with prostate cancer, related to reduced survival, is a rising titer of serum prostate-specific antigen (PSA) after an initial response to androgen deprivation. The transcriptional regulation of the PSA gene has been shown to correlate well with the progression of prostate cancer, with both gene expression and the disease going from an androgen-dependent to an androgen independent stage. Once the malignancy is androgen-independent it is able to grow in the absence of androgen and at present no therapy can be offered to the patient. Therefore, it is critically important to identify the underlying molecular mechanisms that enable prostate cancer cells to grow in the absence of androgen such that new therapies can be developed. However, investigation of these mechanisms has been impeded by the lack of an *in vivo* model that yields uncontaminated tumors. Available human xenograph models that progress to androgen independence after castration of the host, yield tumors that are highly contaminated with host cells. Therefore the purpose of this work is to develop an *in vivo* model that encompasses the use of hollow fibers to retrieve uncontaminated packages of prostate cancer cells (tumors) that can be used for subsequent molecular biology analyses. These packages, or tumors, of uncontaminated human prostate cancer cells will be harvested from the animal both prior to, and subsequent to, the onset of androgen-independent tumor progression as indicated by serum levels of PSA. Thus, our specific aims are the following. **1)** To characterize the growth and progression to androgen-independence of human LNCaP prostate cancer cells grown in hollow fibers and maintained *in vivo* in immunocompromised mice. **2)** To identify the differential expression of known and unknown genes by application of cDNA arrays and PCR-based subtractive hybridization of androgen-sensitive versus androgen-independent cells using this *in vivo* hollow fiber model. We anticipate that the proposed research will provide new insight into the molecular basis of progression to androgen-independence and identify new targets for delaying/averting androgen resistance in prostate cancer.

BODY

The foundation of the proposed research is based upon the fact that androgen-independent increases in serum PSA of patients with prostate cancer correspond to the emergence of androgen-independent disease. Therefore identifying the changes in gene expression in prostate cancer cells associated with progression to androgen-independent disease, as indicated by serum PSA, should identify molecular targets for therapeutic intervention. The following work describes our progress over the entire funding period for the development and characterization of a new animal model for the study of the molecular events underlying progression of prostate cancer to androgen-independence. The strength of this model is that it restricts the infiltration of contaminating host cells thereby yielding "pure" populations of prostate cancer cells that can be used for molecular analysis.

Task 1 - Characterization of the growth of human prostate cancer cells maintained *in vivo* inside of hollow fibers (months 1-18): completed and the results have been published in Molecular Cancer Therapetutics (see attached manuscript in appendix).

In vivo cultivation of LNCaP human prostate cancer cells in hollow fibers – Elevation of levels of PSA in the serum of men with prostate cancer has been suggested to be directly correlated to the tumour volume (1,2). To test whether LNCaP cells grown in fibers could proliferate, we used the reported optimal seeding density for other cell lines that was $1x10^7$ cells/ml (3) and measured serum levels of PSA in the implanted mice. Male Nude mice prior to implantation with fibers containing LNCaP cells had undetectable levels of serum PSA (< 0.02 ng /ml of serum) as expected since PSA is a human specific protein that is secreted by prostate epithelial cells (4). At a cell density of $1x10^7$ cells/ml, a continuous rise in serum PSA levels were measured over the duration of the experiment (Fig. 2 of the attached manuscript). Fourteen days after implantation of the fibers, serum PSA levels were 4 ng/ml. These PSA levels continued to rise to approximately 16 ng/ml by day 56 after implantation of the fibers. Thus a 4-fold increase in serum PSA levels were measured in the intact animal over the period of 42 days giving a velocity of 2 ng/ml/week. Serum PSA values obtained from a parallel set of mice that were seeded with a higher density of LNCaP cells ($1x10^8$ cells/ml) showed an initial sharp rise followed by a plateau. Fourteen days after implantation of fibers containing LNCaP cells, a mean serum PSA level of 10 ng/ml was detected (Fig. 2 of the attached manuscript). By day 28, serum PSA had continued to rise to a level of 25 ng/ml, representing a velocity of 7.5 ng/ml/week. By day 42 no further rises in serum PSA were observed.

Inclusion of Matrigel® is not required for the LNCaP hollow fiber model – Xenograph experiments in Nude and SCID mice require the inclusion of Matrigel® in order for LNCaP cells to form tumors (5). To test whether Matrigel® had an effect on proliferation of LNCaP cells grown hollow fiber model we examined the levels of serum PSA in the Nude mice containing: 1) LNCaP cells without Matrigel® and pre-seeded 3 days in fibers maintained *in vitro* before implantation into mice; 2) LNCaP cells not pre-seeded and without Matrigel®; or 3) LNCaP cells not pre-seeded in the presence of Matrigel®. Serum PSA levels were comparable between the animals containing fibers that were not pre-seeded, regardless of the inclusion or absence of Matrigel® (Fig. 3 of the attached manuscript). Serum PSA levels were approximately 7.5 ng/ml at 7 days after implantation of fibers containing LNCaP cells either with or without Matrigel®. Serum PSA levels were lower in the mice containing fibers that were pre-seeded 3 days earlier and maintained *in vitro* before implantation. However, by 2 weeks after implantation there

was no difference in serum PSA levels between the 3 groups of animals. All three sets of animals responded similarly to castration with a greater than 85% drop in serum PSA by one week after castration. All three sets of animals also showed androgen-independent increases in serum PSA levels over the nadir at 4 weeks after castration. These results suggest that Matrigel® is not required in order for LNCaP cells to grow, respond to androgen, and become androgen-independent inside hollow fibers maintained *in vivo* as determined by serum PSA responses.

Androgen-independent increases in serum PSA after castration – Androgen deprivation therapy in most patients with prostate cancer results in an 80% drop in serum PSA levels (6). Here we examined the effects of androgen deprivation on serum PSA levels in mice implanted with fibres containing LNCaP cells that were surgically castrated after serum PSA levels were shown to be stable or rising. After castration, serum PSA levels were monitored for an additional 14 weeks. Results from 3 separate experiments (4 Nude mice per experiment) showed that castration resulted in a 80% drop in serum PSA levels by 2 weeks post-castration (Fig. 4 of the attached manuscript). Four weeks post-castration serum PSA levels rose again, signifying the emergence of androgen-independence. By approximately 14 weeks post-castration, serum PSA levels were increased by 3.5-fold over the PSA nadir.

PSA mRNA levels correspond to serum PSA levels - Using the LNCaP xenograph model of prostate cancer, increases in PSA gene expression in androgen-independent cells is established at the level of transcription (7). Both serum PSA and tumor mRNA levels are down-regulated when testosterone is withdrawn, and up-regulated when it is replaced. However, when the tumor becomes androgen-independent, PSA mRNA is constitutively up-regulated despite the continuing absence of testosterone. To test whether this occurs in LNCaP hollow fiber model, we performed Northern blot analyses on LNCaP cells harvested from fibers in SCID mice at the following time points: 1) day 5 when the serum PSA was elevated prior to castration; 2) four days after castration when the serum PSA was dropping; and 3) twenty-four days after castration when the serum PSA was elevated (Fig. 6A of the attached manuscript). Results from Northern blot analyses of RNA isolated from LNCaP cells harvested at these time points in a single animal are shown in Fig. 6B of the attached manuscript. Northern blot analysis of PSA mRNA levels from 3 to 4 different animals, normalized with 18S RNA and bands quantified by phosphorimaging are shown in Fig. 6C of the attached manuscript. These data indicate that PSA mRNA levels are: 1) elevated in the presence of androgen at time point 1 (Intact) which corresponds to elevated serum PSA at this time point; 2) decreased at time point 2 (CX), in the absence of androgen, when serum PSA levels are dropping; and 3) elevated in the absence of androgen (AI) when serum PSA levels are elevated signifying androgen-independence.

Cell growth, doubling time, and viability assays – One of the most beneficial aspects of this model is that cell number within fibers can be determined at any point in the experiment. To determine if LNCaP cells proliferate in this model we assessed the seeding and harvested cell densities using the MTT assay to calculate the net % growth (3). In the group of intact animals, cells seeded at a density of $1x10^7$ cells/ml continued to proliferate over the duration of the experiment at 48 days after implantation of the fibers (Fig. 7A of the attached manuscript). The rate of proliferation was relatively constant during this time and 200% net growth was calculated at the duration of the experiment. In the group of animals that were castrated at 21 days after implantation (Fig, 7B of the attached manuscript), the initial rate of proliferation in the presence of testosterone was similar to that observed in the intact animals as would be expected (Fig. 7A of the attached manuscript). However after castration, the rate of proliferation was

markedly decreased as indicated by the change in slope after 21 days (Fig. 7B of the attached manuscript)). A reduced net % growth of 132% was calculated for LNCaP cells maintained in animals castrated at day 21 at the duration of the experiment, as compared to 200% for the intact animals.

Response to intermittent androgen suppression - In approximately 30% of animals, the cells grown in fibers failed to become androgen-independent after extended periods in the castrated host. To test whether this was due to the loss of cell viability from possibly necrosis or apoptosis, we inserted a testosterone pellet subcutaneously to determine if the cells would still respond to androgens after these lengthy periods of cultivation in the animals. Results presented in Fig. 8A of the attached mansucript show that in Nude animals that did not become androgen-independent 10 weeks after castration, the implanted LNCaP cells still responded to testosterone as indicated by the rapid and robust increase in serum PSA to values greater than pre-castrate levels. Upon removal of the testosterone pellet, serum PSA levels dropped by greater than 90%. Similarly, LNCaP cells maintained in fibers in SCID mice that did not become androgen-independent 7 weeks after castration still responded robustly to testosterone as indicated by the 2-fold increase in serum PSA levels over pre-castrate values (Fig. 8B of the attached manuscript). Animals that did not become androgen-independent were next examined to test their response to several cycles of androgen-withdrawal and -replacement. When the testosterone pellets were inserted, each of the animals responded with a robust increase in serum PSA levels above pre-castrate values (Fig. 8C of the attached manuscript). When the testosterone pellet was removed, serum PSA returned to nadir levels. Serum PSA response to testosterone could be observed over numerous intervals reflecting the clinical scenario observed in patients treated with intermittent androgen suppression (8).

Tumor morphology and histology - Harvested fibers containing cells were fixed in 10% buffered formalin, embedded in paraffin, sectioned (longitudinal and cross-section), and stained with hematoxylin and eosin to evaluate the cells cultivated within the fiber. These studies demonstrated that initially the cells grew along the wall of the fiber. In intact animals the cells tended to stack-up forming layers and scaffold structures resembling a solid tumor with areas that contained necrotic populations (Fig. 9A of the attached manuscript). Cells grown in the presence of Matrigel® and maintained *in vivo* in fibers is also shown (Fig. 9B of the attached manuscript). Surprisingly, often fibers removed from castrated animals with elevated levels of serum PSA contained spheroids of cells that had detached from the fiber wall (Fig. 9C of the attached manuscript).

Re-culturing cells - One potential application of this model is to develop new sublines of cells with different requirements for androgen. Presently there limited prostate cancer cell lines that are androgen-independent and still express androgen receptor (9). To develop an such a cell line using the hollow fiber model, androgen-independent cells were harvested from castrated animals with increasing serum PSA and recultured and maintained *in vitro*. While re-culturing *in vitro* in RPMI-medium supplemented with 10% DCC-FBS, many cells did not attach to the plate surface and clumps of viable cells floated on the surface of the medium. However, when the cells were able to attach and grow, they resembled neurons, with a small cell body and long dendrite-like outgrowths (Fig. 10 of the attached manuscript).

Summary of Task 1

Development of LNCaP hollow fiber model for the study of progression of prostate cancer to androgen–independence is shown here for the first time. In the course of developing this model several important observations were made. These include: 1) Matrigel® and direct cell-cell interactions between LNCaP

and stroma cells are not required for proliferation or progression of LNCaP cells to androgen-independence; and 2) androgen-independent increases in serum PSA in castrated animals is uncoupled from proliferation.

Task 2 - Molecular analyses of human prostate cancer cells maintained *in vivo* inside of hollow fibers and harvested at different stages of progression to androgen independence (months 8-36): completed.

The ultimate objective of these experiments is to develop a model that provides "pure" populations of cells for the molecular analysis of the changes in gene expression that occur in the progression to androgen-independence. Now that this model has been established we have examined differences in gene expression by commercial cDNA arrays and subtractive hybridization.

***Subtractive hybridization*- Completed**. Through the technique of subtractive hybridization we have created a library of genes that are differentially expressed at specific time points during progression when compared to cells isolated from non-castrated mice. A total of six subtractive hybridizations in three experimental groups were performed with each using RNA from intact mice as a control. These experimental groups are shown in Figure 1 (below) and include:

1) androgen dependent cells 10 days post-castration relative to intact;
2) androgen independent cells 60 days post-castration relative to intact; and
3) androgen dependent cells 61 days post-castration relative to intact.

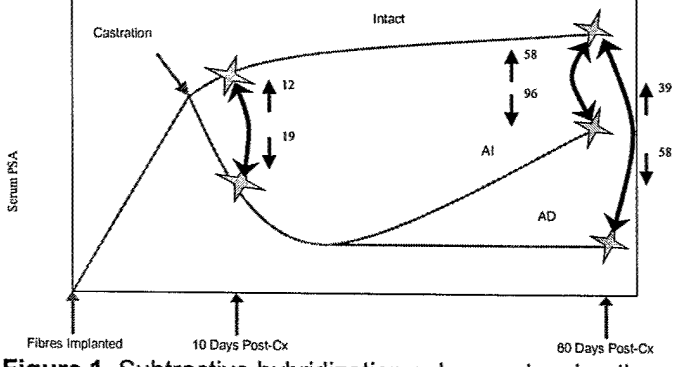

Figure 1. Subtractive hybridization scheme showing the harvest times and points compared. The numbers next to the arrows represent the number of clones either up- or down-regulated compared to cells harvested from the intact animal at that time point.

A total of 350 clones were isolated using this method and each of these clones was sequenced unidirectionally. Database searches were carried out for 267 clones (those for which sufficient sequence data was available) to identify the gene present in each clone. Initially, a high stringency, automated search was performed of the known and predicted full-length cDNAs present in the Mammalian Gene Collection (MGC), Ensembl predicted cDNAs, and the Human UniGene Clusters. Full-length cDNAs producing alignments meeting specific threshold criteria were searched in the SWall (SPTR) protein database to identify proteins associated with each cDNA. This database search provided preliminary information concerning functionality for each of the protein matches.

Of the original 267 clones, 104 did not match full-length cDNAs. These clones were mapped to the human genome by comparing the results from three different, on-line, database searches at the Ensembl Human Genome Server, the Human Genome Project Working Draft at UCSC, and The Human Genome at NCBI. A clone was considered successfully mapped if the results of each of these searches were consistent with one another. In these cases, information regarding known and predicted genes in these regions of the genome was extracted. The identified genes were divided into four functional groups (housekeeping, gene expression, signal transduction, and unknown) each consisting of several sub-groups. For housekeeping genes these included ribosomal proteins, metabolism, catabolism,

replication, cytoskeleton, ubiquitination, and protein trafficking. For genes involved in gene expression these included transcription, translation, DNA-binding, and chromatin regulation. For genes involved in signal transduction these included kinase phosphatases, receptors, chaperones, ligands, and Ca^{2+}/phospholipid binding.

In the 10 day castrated animals that had low serum PSA values, 12 clones (4 non-redundant sequences) were up-regulated and 19 clones (7 non-redundant sequences) were down-regulated in the cells harvested from the fibers as compared those harvested from the intact (high PSA) animal. The breakdown of the function of these genes are shown in Figures 2 and 3.

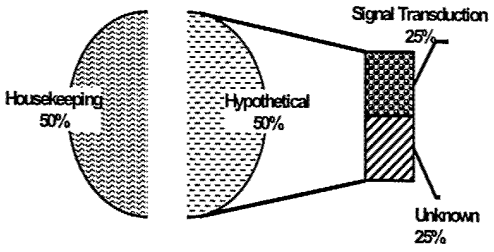

Figure 2. Functional distribution of up-regulated sequences isolated at 10 days post-castration (low serum PSA levels) relative to intact.

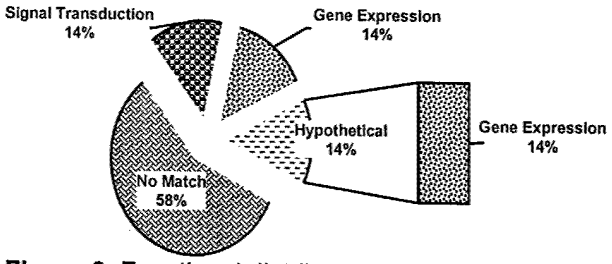

Figure 3. Functional distribution of down-regulated sequences isolated at 10 days post-castration (low serum PSA levels) relative to intact.

In the 60 day castrated animals that had a high serum PSA signifying androgen-independence, 58 clones (38 non-redundant) were up-regulated and 96 clones (44 non-redundant sequences) were down-regulated in the cells harvested from the fibers as compared those harvested on the same day from the intact (high PSA) animal. The breakdown of the function of these genes are shown in Figures 4 and 5.

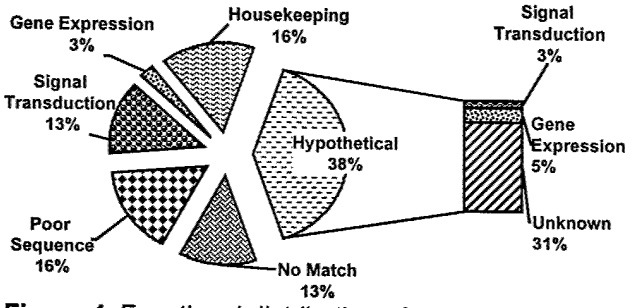

Figure 4. Functional distribution of up-regulated, sequences isolated from androgen-independent LNCaP cells (high PSA in castrated animals) relative to LNCaP cells harvested from intact animals.

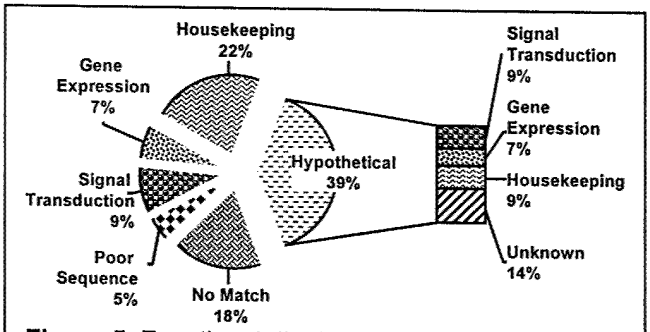

Figure 5. Functional distribution of down-regulated, sequences isolated from androgen-independent LNCaP cells (high PSA in castrated animals) relative to LNCaP cells harvested from intact animals.

In the 60 day castrated animals that had a low serum PSA, 39 clones (23 non-redundant) were up-regulated and 58 clones (30 non-redundant sequences) were down-regulated in the cells harvested from the fibers as compared those harvested on the same day from the intact (high PSA) animal. The breakdown of the function of these genes are shown in Figures 6 and 7.

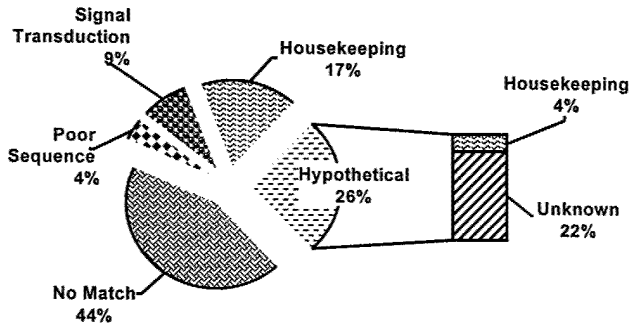

Figure 6. Functional distribution of up-regulated sequences isolated from androgen-dependent cells (low PSA in castrated animals) relative to LNCaP cells harvested from intact animals.

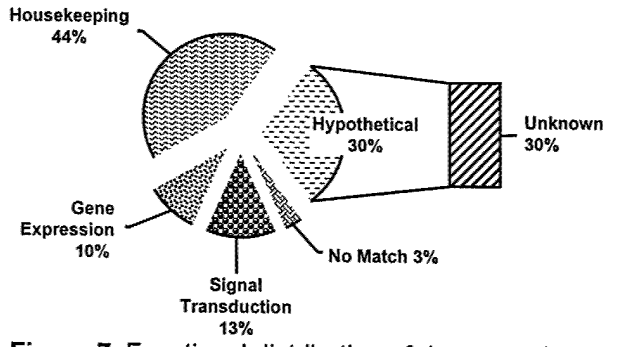

Figure 7. Functional distribution of down-regulated sequences isolated from androgen-dependent cells (low PSA in castrated animals) relative to LNCaP cells harvested from intact animals.

In conclusion, 146 non-redundant genes that are differentially expressed during progression to androgen independence have been isolated using subtractive hybridization and sequencing technology. Approximately one-half of these genes (77 genes) have unknown function. 156 different clones that include some controls isolated from the subtractive hybridization experiment have been spotted onto a custom array along with approximately known genes and are currently being used to screen a large number of samples.

Commercial arrays - **Completed**. Using Clontech Atlas Human Cancer cDNA Arrays we determined the change in gene expression in animals just prior to castration (Pre-CX; five days after implantation), four days after castration (CX-AD; 9 days after implantation), and in androgen-independent cells harvested 24 days after castration when the serum PSA had risen above nadir values (CX-AI; 29 days after implantation). Examples of genes previously reported to be altered with castration and androgen-independence are shown in Figure 8.

Recently Gregory et al (10) reported that cdk1 and cdk2 down-regulated with androgen withdrawal and increased in androgen-independence while cdk4 does not change. In our LNCaP hollow fiber model we showed that cdk1 is not altered while cdk2 increased with castration and continued to increase in androgen-independent cells (Figure 8A). Cyclin B1 did not significantly change, while cyclin D1 was decreased in castrated animals and androgen-independent animals. Cdc4 was not significantly altered in castrated animals while it appeared to decrease in androgen-independent animals. Consistent with previous reports, phospholipase D1 increased with castration and in androgen-independent cells (11).

Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) are thought to play an important role in prostate cancer. Castration caused a decrease in EGF which is consistent with previous reports (Figure 8B). Amphiregulin, heparin binding-EGF (HB-EGF), TGF, TGF-α, TGF-β1, and TGF-β3 increased with castration. However, only amphiregulin, HB-EGF, TGF, and TGF-β3 were also increased in androgen-independence compared to cells harvested from intact animals. The insulin-like growth factors (IGF) and axis was also altered. IGF binding protein-2 (IGFBP-2) has been shown to be over-expressed in 100% of hormone-refractory tumors (12). In our model, IGFBP2 and IGFBP-3 were increased in androgen-independent cells while IGFBP4, IGFBP5, and IGFBP6 were relatively unchanged. IGF1 receptor (IGF1R) decreased in androgen-independent cells. Tumor necrosis factor receptor 1β (TNF1B) increased more than two-fold after castration. VEGF decreased with

castration while VEGFB showed no change. VEGFC increased only with androgen-independence. Interleukin-6 (IL-6) was highly expressed and increased in castrated and androgen-independent cells.

In Figure 8C, BAG-1 increased with castration and androgen-independence. BAX showed no change while MCL1 increased with castration. Of the caspases measured, caspase 6 and caspase 9 increased after castration and then returned to intact levels in the androgen-independent cells. Of the matrix metalloproteinases (MMPs), MMP7 increased with castration while MMP8 only slightly increased, and MMP9 slightly decreased in androgen independent cells. TIMP1 and TIMP2 increased in androgen-independent cells compared to levels in castrated animals.

Prostate-specific membrane antigen, vimentin, caveolin-1, fibronectin (13), versican, and E2A ubiquitin-conjugating enzyme (13) increased with castration while plasminogen decreased which is consistent with previous reports (Figure 8D).

While this cDNA array data is only a small amount of the total data that has been generated, it emphasizes that using RNA isolated from the LNCaP hollow fiber model produces results that are consistent with changes in gene expression described for clinical samples and other models of androgen independence.

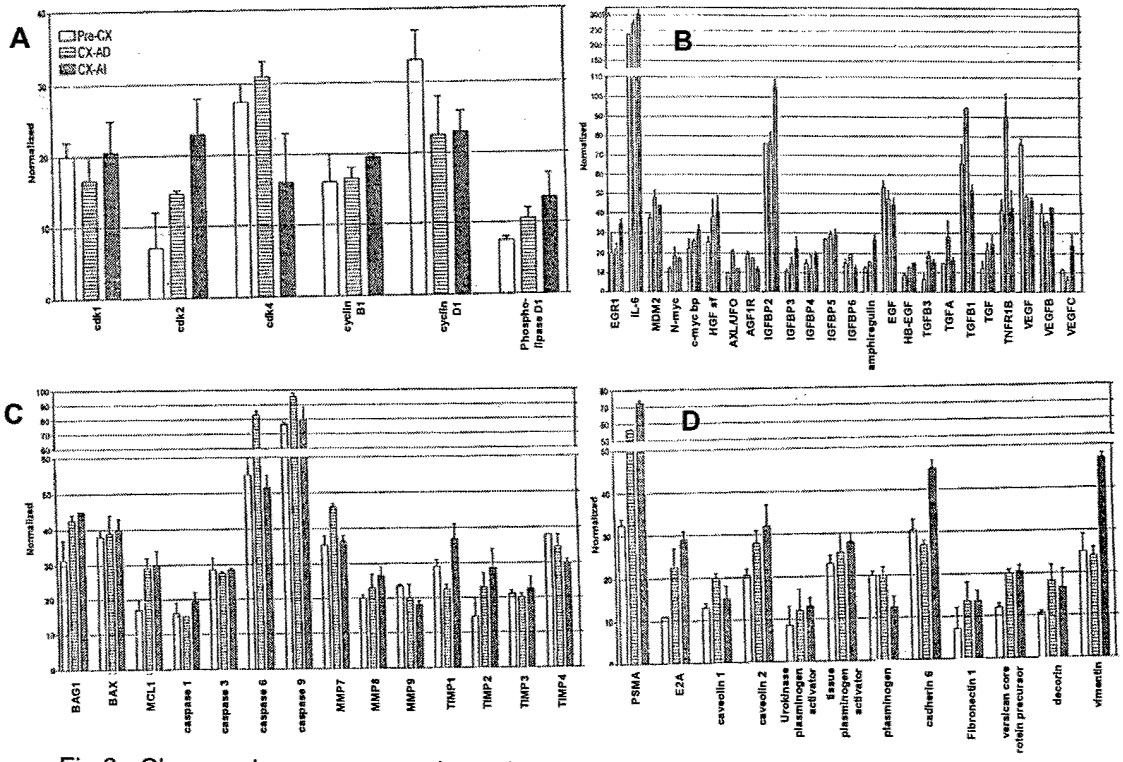

Fig 8. Changes in gene expression using the LNCaP Hollow Fiber Model. RNA was extracted from fibers harvested at the indicated times, radiolabeled with P^{32} and hybridized with Clontech Atlas Human Cancer cDNA Arrays. The arrays were quantified using a phosphorimager, analyzed using Imagene software, and the bands normalized to a housekeeping gene on each array (myelin basic 23). Pre-CX: animals just prior to castration (five days after implantation). CX-AD: four days after castration (9 days after implantation). CX-AI: androgen-independent cells harvested 24 days after castration when the serum PSA had risen above nadir values (29 days after implantation).

Validation of changes in gene expression. With the isolation of so many known (69) and unknown genes (77), it was overwhelming to perform Northern blots for validation of expression of each gene. Since so many genes were completely unknown there were no commercial antibodies available and thus we could not perform Western blots. We did however, use Northern blot analysis to validate expression of a few of the unknown genes that were randomly chosen. Fig. 9 shows that the expression of clones 2F10 and 1C06 are up-regulated by dihydrotestosterone (DHT) when RNA is isolated from LNCaP cells maintained in culture. Clone 1F10 was shown by Northern blot to be up-regulated by DHT *in vitro* and in AI cells *in vivo* (Fig. 10). A BLAST search of clone 1F10 in Ensembl Human Genome Database is shown in Fig. 11 and did not match any known or predicted exons. Clone 1F10 may represent a splice variant of a novel gene with no known function.

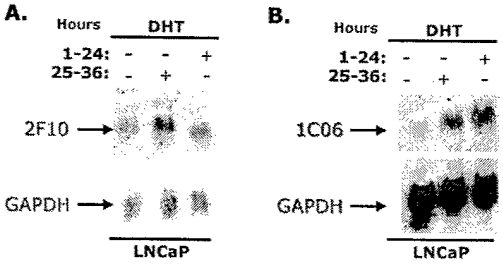

Fig. 9. Northern blots using probes to clones 2F10 (A) and 1C06 (B) against total RNA isolated from *in vitro* experiments. LNCaP cells were grown with (+) or without (-) dihydrotestosterone (DHT) for the indicated times.

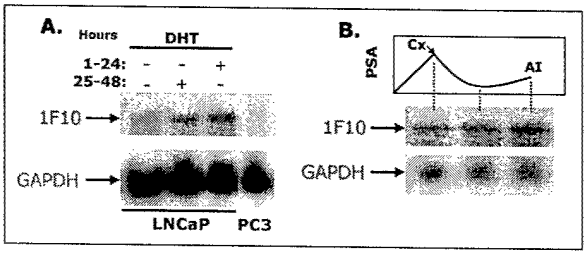

Fig. 10. Northern blots using a probe to clone 1F10 against total RNA isolated from *in vitro* experiments (*A*) and from the hollow fibre model (*B*). *A*. LNCaP cells were grown with (+) or without (-) dihydrotestosterone (DHT) for the indicated times. Total RNA was also isolated from PC3 cells. *B*. Total RNA was isolated from fibres in one mouse on the day of castration, 10 days post-castration and in androgen-independence.

Thus we have shown that indeed these handful of genes are expressed and up-regulated in response to treatment with DHT. A cost effective, and sample conserving approach to validate the expression of so many genes was begun just prior to the termination of funding. This involved the development of our own custom microarray chip that will be spotted with 156 clones from the subtractive hybridizations experiments (including controls) and 107 known genes that have either been reported in the literature to show altered expression in androgen independent

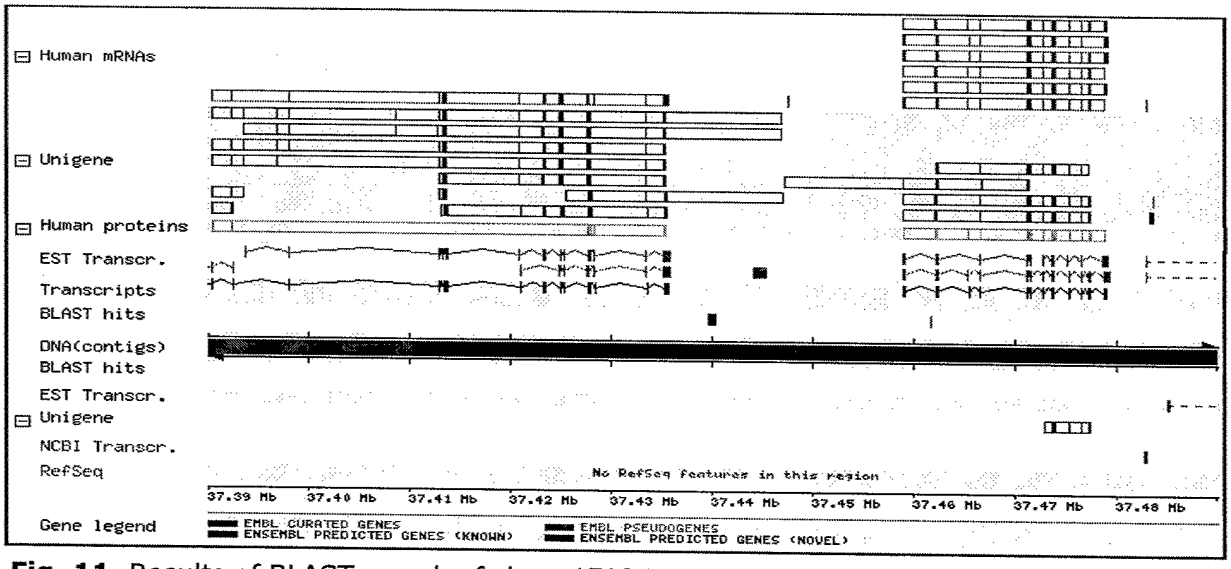

Fig. 11. Results of BLAST search of clone 1F10 in Ensembl Human Genome Database. This clone was up-regulated in AI relative to Intact and does not match any known or predicted

disease or were shown to be altered when samples were probed on the Clontech cDNA array. This custom microarray chip will be repeatedly screened using RNA from *in vitro* and *in vivo* samples to provide validation for the results presented here. These chips will also be screened using patient samples to examine the possibility of using these chips as a diagnostic or prognostic tool. The information gained from these expression profiles will ultimately be used to identify new therapeutic targets for the treatment of androgen-independent prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Characterization of the LNCaP hollow fiber model that progresses to androgen independence.
- Matrigel® and direct cell-cell interactions between LNCaP and stroma cells are not required for proliferation or progression of LNCaP cells to androgen-independence.
- Androgen-independent increases in serum PSA in castrated animals is uncoupled from proliferation.
- Screening of RNA isolated from cells during progression to androgen independence using cDNA arrays containing known genes has shown that changes in gene expression in this model corresponds to that reported in the literature for clinical specimens and other models of androgen-independence. This validates that changes in gene expression in the model mimics that known in clinical disease and that more genes besides PSA become elevated with progression to androgen independence.
- Subtractive hybridization experiments have isolated 146 non-redundant genes of which 77 genes have unknown function. Of the 77 genes with unknown function, 28 of these genes had no match when searched at the Ensembl Human Genome Server, the Human Genome Project Working Draft at UCSC, and The Human Genome at NCBI.
- Validation of randomly chosen genes of unknown function showed expression and up-regulation in LNCaP cells maintained in vitro in response to androgens when using Northern blot analysis.
- Clones have been prepared for spotting our own custom microarray for the screening of RNA isolated from prostate cancer cells maintained *in vitro* and *in vivo* samples as well as clinical samples from biopsy and radical prostatectomy.

REPORTABLE OUTCOMES

Manuscripts

1. Sadar, M.D., Akopian, V., and Beraldi, E. (2002) Characterization of a new *in vivo* hollow fiber model for the study of progression of prostate cancer to androgen independence. *Molec. Cancer Therap.*, 1, 629-637.

Abstracts

1. Quayle, S., Hare, H., Akopian, V., Hwang, D., Jones, S., Schein, J., Marra, M., and Sadar, M.D. Discovery of new genes differentially expressed in androgen independent prostate cancer. *Annual BC Cancer Agency Clinical Cancer Conference*, November 29-30, 2002, Vancouver, Canada.
2. Sadar, M.D., Akopian, V., and Beraldi, E. Characterization of a new *in vivo* hollow fiber model for the study of progression of prostate cancer to androgen-independence. *Endocrine Society's 84th Annual Meeting*, June 19-22, 2002, San Francisco, USA.
3. Quayle, S., Hare, H., Akopian, V., Hwang, D. Jones, S., Schein, J., Marra, M., and Sadar, M. Gene expression profiles associated with progression of prostate cancer to androgen-independence. *Endocrine Society's 84th Annual Meeting*, June 19-22, 2002, San Francisco, USA.
4. Quayle, S., Hare, H., Akopian, V., Hwang, D., Jones, S., Schein, J., Marra, M., and Sadar, M. Identification of a novel gene differentially expressed in the progression of prostate cancer. *Pathology Day, Department of Pathology and Laboratory Medicine, University of British Columbia*, May 10, 2002, Vancouver, Canada.
5. Quayle, S., Hare, H., Akopian, V., Jones, S., Schein, J., Marra, M., and Sadar, M. Gene expression analysis of androgen-independent prostate cancer. *Northwest Urological Society Meeting 2001*, December 7-8, 2001, Vancouver, Canada.
6. Akopian, V., Beraldi, E., and Sadar, M. Characterisation of a new *in vivo* model for genomic and proteomic analyses of progression of prostate cancer to androgen-independence. *Northwest Urological Society Meeting 2001*, December 7-8, 2001, Vancouver, Canada.
7. *Quayle, S., Hare, H., Akopian, V., Jones, S., Schein, J., Marra, M., and Sadar, M. Differential gene expression in a model of advanced prostate cancer. *Annual BC Cancer Agency Clinical Cancer Conference*, November 23-24, 2001, 2001, Vancouver, Canada. **Best poster Award**.
8. Quayle, S., Hare, H., Akopian, V., Jones, S., Schein, J., Marra, M., and Sadar, M. Identification of new targets for the treatment of androgen-independent prostate cancer. *Functional Genomics*, Satellite Meeting of the 8th ICEM, October 16-18, 2001, Seattle, Washington.
9. Adomat, H., Akopian V., Ma, S., Diamond, D., Webber, D., Vielkind, J., and Sadar, M. Application of surface enhanced laser desorption/ionization technology for the study of prostate cancer progression. *92nd Annual AACR Meeting*, March 24-28, 2001, New Orleans, LA.
10. Akopian, V., Bruchovsky, N., Beraldi, E., Signaevsky, M. and Sadar, M.D. Characterization of a new *in vivo* prostate tumor model. *9th Annual Society for Basic Urologic Research (SBUR) Fall Symposium*, Nov. 9-12, 2000, Fort Myers, FL.

Presentations

1. **Department of Urology, Northwestern University Medical School.** *Prostate Cancer: Unravelling the molecular mechanisms underlying progression to androgen independence.* Chicago, November 26, 2002.
2. **University of Texas Health Science Center San Antonio,** Division of Medical Oncology. *"Mechanisms underlying the progression of prostate cancer to androgen independence".* San Antonio, Texas, August 28, 2002.
3. **ENDO 2002, Endocrine Society's 84th Annual Meeting.** *Characterization of a new in vivo hollow fiber model for the study of progression of prostate cancer to androgen-independence.* San Francisco, USA, June 19, 2002.
4. **Molecular and Cellular Biology Research Seminar Series.** *"Mechanisms underlying the progression of prostate cancer to androgen independence".* Sunnybrook and Women's College Health Sciences Centre, Toronto, Ontario, February 27, 2002.
5. **Annual BC Cancer Agency Clinical Cancer Conference.** *"Mechanisms underlying the progression of prostate cancer to androgen independence".* Vancouver, November 23, 2001.
6. **McGill Cancer Centre.** *ProteinChip Technology Facilitates an Integrated Approach to the Study of Prostate Cancer."* Montreal, Quebec, Nov. 21, 2001.
7. **University Health Network, Toronto General Hospital.** *ProteinChip Technology Facilitates an Integrated Approach to the Study of Prostate Cancer."* Toronto, Ontario, Nov. 19, 2001.
8. **Universitätsklinik für Urologie.** *"Mechanisms underlying the progression of prostate cancer to androgen independence".* Innsbruck, Austria, October 29, 2001.
9. **St. Pauls' Hospital - ICAPTURE.** *"Ciphergen ProteinChip Array Technology Facilitates an Integrated Approach to the Study of Prostate Cancer."* Vancouver, Sept. 11, 2001.

- **patents and licenses applied for and/or issued:** none yet
- **degrees obtained that are supported by this award:** Steven Quayle (full salary scholarship from other sources) worked this project as part of his PhD thesis (Dept. of Pathology, University of British Columbia, Vancouver, Canada).
- **development of cell lines, tissue or serum repositories**: none
- **informatics such as databases and animal models, etc**: This grant is the development of an animal model.
- **funding applied for based on work supported by this award:** none yet
- **employment or research opportunities applied for and/or received on experiences/training supported by this award:** Dr. Viola Akopian (Post-doctoral fellow: 2000-2001); Dr. Katie Meehan (Post-doctoral fellow, 2002); Heidi Hare (Research Assistant); Sherman Tung, Michelle Joe, and Tammy Romanuik (undergraduate students).

CONCLUSIONS

Work undertaken has led to the development of a new hollow fiber model for the study of prostate cancer progression. The well-characterized LNCaP xenograph tumor model grows in male Nude mice and secretes PSA into the serum (14-16). Upon castration, the serum PSA drops, but after 4 weeks PSA expression increases above pre-castrate levels signifying androgen-independent regulation of PSA (14-16). Similarly, the LNCaP hollow fiber model follows the same trend as the LNCaP xenograph tumor model in its progression to androgen-independence and regulation of serum PSA and mRNA levels. Therefore, this fiber model has demonstrated that androgen-independence can occur in LNCaP cells maintained *in vivo* without direct cell-cell interaction with the stroma, nor is the inclusion of Matrigel® required as suggested for the LNCaP xenograph model (14). This model has generated data from cDNA arrays and subtractive hybridization using RNA isolated from LNCaP cells harvested before and after castration of the host, during various stages of progression to androgen-independence. With the aid of cDNA it was shown that many of the genes altered with castration and androgen-independence in this hollow fiber model were consistent to those previously reported using other models or patient tissue thereby validating the relevance of using the hollow fiber model for studying progression of prostate cancer to androgen independence. Subtractive hybridization experiments have isolated 146 non-redundant genes of which 77 genes have unknown function. Of the 77 genes with unknown function, 28 of these genes had no match when searched at the Ensembl Human Genome Server, the Human Genome Project Working Draft at UCSC, and The Human Genome at NCBI. Expression of some of the unknown genes was confirmed by Northern blot analysis and showed that expression of these genes was up-regulated by androgens. The clones isolated from the subtractive hybridization will be spotted as a custom microarray chip to screen RNA from *in vitro* and *in vivo* samples. This will provide validation for the results presented here. These chips will also be screened using patient samples to examine the possibility of using these chips as a diagnostic or prognostic tool. The information gained from these expression profiles will ultimately be used to identify new therapeutic targets for the treatment of androgen-independent prostate cancer.

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APPENDICES

1. **Sadar**, M.D., Akopian, V., and Beraldi, E. (2002) Characterization of a new *in vivo* hollow fiber model for the study of progression of prostate cancer to androgen independence. *Molec. Cancer Therap.*, 1, 629-637.

Characterization of a New *in Vivo* Hollow Fiber Model for the Study of Progression of Prostate Cancer to Androgen Independence[1]

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Abstract

Research investigating the molecular events underlying progression of prostate cancer to androgen independence has been impeded by the lack of an appropriate *in vivo* model that yields "pure" populations of prostate cancer cells that are not contaminated with host cells. Here we characterize a new *in vivo* model that uses hollow fibers and allows for the retrieval of uncontaminated prostate cancer cells during various stages of endocrine progression to androgen independence in male immunocompromised mice. Prostate-specific antigen (PSA) gene expression, proliferation of cells, and histology were examined in these mice before and after castration. LNCaP cells seeded at a density of 1×10^7 cells/ml, or a total of approximately 4.8×10^6 cells/animal, provided measurable serum PSA levels that increased in intact (noncastrated) animals, decreased by 80% to a nadir after castration, and subsequently increased by 4 weeks after castration, indicating progression to androgen independence. *In vivo* proliferation of LNCaP cells inside the fibers continued in the presence of androgens and continued to increase, albeit at a slower rate, in the castrated animals. Histology of cells cultivated in hollow fibers demonstrated that initially the cells grew along the wall of the fiber and tended to stack up, forming layers and scaffold structures resembling a solid tumor. Fibers removed from castrated animals with elevated levels of serum PSA contained spheroids of cells that had detached from the fiber wall. The development of the LNCaP hollow fiber model described here provides a reproducible means of obtaining "pure" populations of LNCaP cells during different stages of progression to androgen independence for molecular analysis requiring RNA and protein extracts free of host cell contamination.

Introduction

Prostate cancer is the second most prevalent cause of death from cancer in American males (1). Localized prostate cancer can be treated surgically or by radiotherapy. The only effective systemic therapy available for metastatic prostate cancer is androgen deprivation. The inability of androgen deprivation to completely and permanently eliminate all prostate cancer cell populations is manifested by the predictable pattern of initial response and relapse with the ultimate progression to androgen independence. Androgen deprivation is associated with a gradual transition of prostate cancer cells through a spectrum of androgen dependence, androgen sensitivity, and ultimately androgen independence.

The tissue-specific marker that is used to monitor treatment responses, prognosis, and progression to androgen independence in patients with prostate cancer is PSA.[3] The levels of serum PSA in most patients is initially androgen regulated and undergo a sharp decline after androgen deprivation. The earliest indication of emerging androgen-independent disease is a rising titer of serum PSA (2–4). Presently, the molecular mechanisms involved in androgen-independent increases in PSA gene expression and the development of androgen-independent prostate cancer are unknown.

Numerous animal models have been developed to investigate the mechanisms underlying the development and pathogenesis of the prostate that include hormone-induced carcinogenesis models, transgenic and reconstitution models, and xenograft models. However, to date no ideal model for prostate cancer exists. This is because the ideal tumor model would have to mimic the clinical presentation, *i.e.,* be of human origin; have a slow doubling time; initially be androgen dependent or respond to androgen; secrete PSA; metastasize to lymph nodes and bone; and hormonally progress to androgen independence after castration (5). Several human xenografts have been developed for the study of prostate cancer including the PC-82 (6), Honda, (7), LuCaP (8), LAP4 and LAPC-9 (9, 10), CWR22 (11), and LNCaP tumor model (12). The LNCaP xenograft can be passaged as a cell line, secretes PSA, and progresses to androgen independence after castration, thereby resembling the pathogenesis of human prostate cancer (12–14). A beneficial aspect of this tumor model is that PSA serum levels can be measured in mice before and after castration to show the transition from androgen sensitivity to androgen independence. However, to determine the molecular events involved in the progression

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[3] The abbreviations used are: PSA, prostate-specific antigen; FBS, fetal bovine serum; SCID, severe combined immunodeficient; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide.

of prostate cancer to androgen independence, homogeneous populations of cells are required for meaningful results. Harvested LNCaP xenografts contain considerable and variable contamination with host tissue. This prevents the use of these tumors for DNA, RNA, and protein extraction. To purify these tumors before they can be used for molecular biology experiments (such as cDNA arrays and proteomics), a labor- and time-intensive protocol is required to isolate the epithelial cells from contaminating host tissue (blood and stroma). Thus, xenograft tumors do not provide an easy model for identifying the molecular events involved in the progression of prostate cancer to androgen independence.

The hollow fiber model was developed by investigators at the National Cancer Institute for *in vivo* cultivation of tumor cells in retrievable packages and prevention of contamination by host cells (15). Although this model has been used for pharmaceutical applications, to date there are no reports of using this model to obtain pure populations of endocrine cells during various stages of hormonal progression. Facilitation of applications using advances in genomic and proteomic technologies requires rapid and pure isolation of tumor cells during a specific stage of tumor development. The aim of this work was to develop an *in vivo* model that encompasses the use of hollow fibers to retrieve uncontaminated packages of prostate cancer LNCaP cells (tumors) that can be used for subsequent molecular biology analyses of the progression of prostate cancer to androgen independence. The manipulation of the tumor cells and the ease with which they can be isolated without host contamination make this model ideal for detailed genomic and proteomic analyses.

Materials and Methods

Animals and Cell Lines. Male athymic Nude mice (BALB/c strain), 6–8 weeks of age, were obtained from Charles River Laboratory (Montreal, Quebec, Canada). Male SCID mice, 6–8 weeks of age, were obtained from the breeding program at the Joint Animal Facility of the British Columbia Cancer Agency. All animals were free of known pathogens at the time of use and maintained in isolator cages. All procedures were performed in compliance with regulations on the humane use and care of laboratory animals under an appropriate animals license issued by the University of British Columbia (Vancouver, British Columbia, Canada). LNCaP cells were kindly provided by Dr. L. W. K. Chung (University of Virginia School of Medicine, Charlottesville, VA) and maintained as a monolayer culture in RPMI 1640 supplemented with 10^5 μg/l penicillin, 10^5 μg/l streptomycin, and 5% FBS (Life Technologies, Inc., Burlington, Ontario, Canada).

Preparation of Hollow Fibers. Polyvinylidene difluoride hollow fibers (M_r 500,000 molecular weight cutoff; 1-mm internal diameter) were purchased from Spectrum Laboratories (Laguna Hills, CA). Preparation of the fibers was performed as described previously (15). Before being filled with LNCaP cell suspensions, the fibers were individually flushed with 70% ethanol and soaked in 70% ethanol for 4 days. After rinsing and autoclaving in deionized water, the fibers were filled with RPMI 1640 supplemented with 20% FBS and incubated at 37°C for 24 h. Subconfluent cultures of LNCaP cells were trypsinized using 0.25% trypsin-EDTA, pelleted by centrifugation, resuspended in RPMI plus 20% FBS, and loaded into the fibers with the aid of a 20-gauge needle at a seeding density of 1×10^7 cells/ml unless stated otherwise. Extremities of the fibers were fused together by heat sealing. Three days after culture *in vitro*, the fibers were sectioned into ~3-cm pieces unless stated otherwise, heat sealed again, and implanted s.c. in Nude or SCID mice.

Implantations and Castration. Mice were anesthetized using methoxyfluorane as an inhalant for all surgical procedures. A small skin incision was made in the hind flank region of the animal to allow the insertion of a 10-gauge trocar. The trocar containing the LNCaP-filled fibers was inserted caudally through the s.c. tissues, thereby allowing the fibers to be deposited during its withdrawal. Unless stated otherwise, a total of 16 fibers were implanted in each animal, in bundles of four fibers at four different regions in the animal. Castration of mice was performed by making a small incision in the scrotum to excise each testicle after ligation of the cord. Surgical suture was used to close the incision. Time of castration varied and is indicated for each experiment.

Intermittent androgen suppression experiments used testosterone pellets (2.5 mg; Innovative Research of America, Sarasota, FL). For each cycle, these pellets were inserted s.c. for 1 week and removed subsequently for 2 weeks, before the cycle was repeated. Serum PSA was monitored over a period of 4 months.

Determination of Serum PSA Levels. Levels of PSA in the blood of implanted animals were determined weekly using an enzymatic immunoassay kit (Abbott IMX, Montreal, Quebec, Canada). Blood samples were obtained by a small incision in the dorsal tail vein to collect ~50 μl of blood using a hematocrit capillary tube. No anesthesia was required. Fifteen μl of mouse serum were diluted with 135 μl of diluent to perform the assay.

Cell Viability and Histology. Fibers were retrieved at different times after implantation, and cell viability was assessed by a modified MTT dye conversion assay as described previously (15, 16).

A parallel set of samples was fixed in 10% buffered formalin and embedded in paraffin. Longitudinal sections of fixed tissue were stained with H&E to evaluate the morphology of LNCaP cells cultivated *in vivo* within the fibers.

Northern Blot Analysis. Total RNA was isolated from LNCaP cells grown inside the fibers using Trizol (Life Technologies, Inc.) and fractionated by electrophoresis on a 1% denaturing agarose gel containing formaldehyde before blotting onto Hybond-N$^+$ filters (Amersham, Oakville, Ontario, Canada). The 1.4-kb *Eco*RI fragment of PSA cDNA (17) and cDNA probe for human glyceraldehyde-3-phosphate dehydrogenase were labeled with [α-^{32}P]dCTP using the Random Primers DNA Labeling kit (Life Technologies, Inc.). Hybridization was performed as described previously (17). The mRNA bands were visualized and quantified using the Storm 860 PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA).

Western Blot Analysis. Nuclear and whole cell proteins (25 μg/lane) were separated by 8% SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The mem-

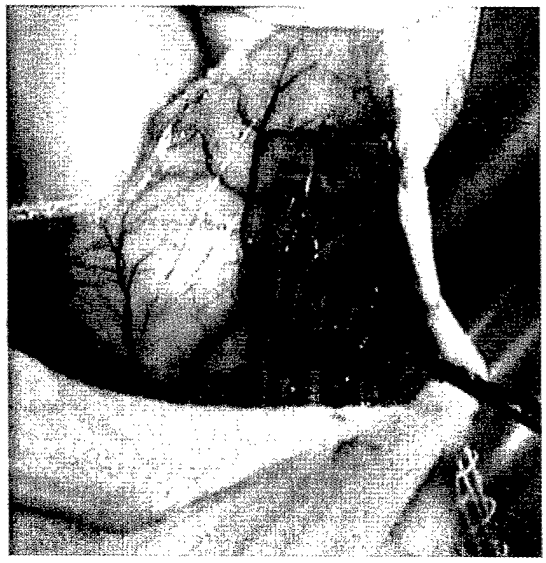

Fig. 1. Morphological appearance of blood vessels in s.c. implanted hollow fibers containing LNCaP cells in a Nude mouse.

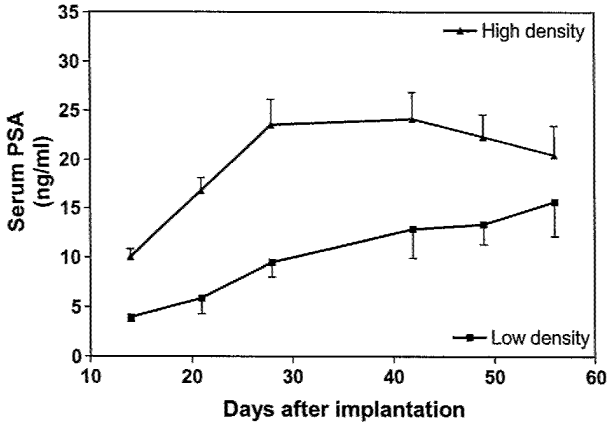

Fig. 2. Serum PSA levels increase with time in noncastrated Nude mice s.c. implanted with fibers containing either 1×10^8 cells/ml (*High density*) or 1×10^7 cells/ml (*Low density*). Data points represent the mean serum PSA ($n = 3$); *bars*, SE.

brane was blocked with 5% (w/v) milk in 20 mM Tris containing 500 mM NaCl and 0.3% Tween 20 (TBST) for 1 h and then incubated 1 h with 0.7 μg/ml antibody to the androgen receptor (SC-7305; Santa Cruz Biotechnology, CA). The membrane was washed and incubated for 1 h with a 1:10,000 dilution of secondary antibody (SC-2005; Santa Cruz Biotechnology). The antibodies were diluted in 5% milk (w/v) in TBST. All incubations were performed at room temperature. Androgen receptor protein was detected using enhanced chemiluminescence kit (Amersham).

Results

Appearance of s.c. Implanted Hollow Fibers Containing LNCaP Cells. The National Cancer Institutes developed the hollow fiber assay to facilitate its screening program for chemotherapeutic agents (15, 18). However, for the chemotherapeutic agents to show efficacy, blood supply to the implanted hollow fibers must be established. In this regard, induction of angiogenesis and development of blood vessels to s.c. implanted hollow fibers has been shown and is dependent on the presence of tumor cells (19). The typical appearance of the fibers containing LNCaP cells that have been implanted s.c. in male Nude mice is shown in Fig. 1.

Cell Density, Proliferation, and Serum PSA. Elevation of levels of PSA in the serum of men with prostate cancer has been suggested to be directly correlated to the tumor volume (20, 21). To test whether LNCaP cells grown in fibers could proliferate, we used the reported optimal seeding density for other cell lines that was 1×10^7 cells/ml (15) and measured serum levels of PSA in the implanted mice. Male Nude mice prior to implantation with fibers containing LNCaP cells had undetectable levels of serum PSA (<0.02 ng/ml of serum) as expected because PSA is a human-specific protein that is secreted by prostate epithelial cells (22). PSA could be detected as early as 3 days after implantation (data not shown), although earlier times were not tested. At a cell density of 1×10^7 cells/ml, a continuous rise in serum PSA levels was measured over the duration of the experiment (Fig. 2). Fourteen days after implantation of the fibers, serum PSA levels were 4 ng/ml. These PSA levels continued to rise to ~16 ng/ml by day 56 after implantation of the fibers. Thus, a 4-fold increase in serum PSA levels were measured in the intact animal over the period of 42 days, giving a velocity of 2 ng/ml/week. Serum PSA values obtained from a parallel set of mice that were seeded with a higher density of LNCaP cells (1×10^8 cells/ml) showed an initial sharp rise, followed by a plateau. Fourteen days after implantation of fibers containing LNCaP cells, a mean serum PSA level of 10 ng/ml was detected (Fig. 2). By day 28, serum PSA had continued to rise to a level of 25 ng/ml, representing a velocity of 7.5 ng/ml/week. By day 42, no further rises in serum PSA were observed.

Inclusion of Matrigel Is Not Required for the LNCaP Hollow Fiber Model. Xenograft experiments in Nude and SCID mice require the inclusion of Matrigel for LNCaP cells to form tumors (14). To test whether Matrigel had an effect on proliferation of LNCaP cells grown in the hollow fiber model, we examined the levels of serum PSA in the Nude mice containing: (*a*) LNCaP cells without Matrigel and preseeded 3 days in fibers maintained *in vitro* before implantation into mice; (*b*) LNCaP cells not preseeded and without Matrigel; or (*c*) LNCaP cells not preseeded in the presence of Matrigel. Serum PSA levels were comparable between the animals containing fibers that were not preseeded, regardless of the inclusion or absence of Matrigel (Fig. 3). Serum PSA levels were ~7.5 ng/ml at 7 days after implantation of fibers containing LNCaP cells either with or without Matrigel. Serum PSA levels were lower in the mice containing fibers that were preseeded 3 days earlier and maintained *in vitro* before implantation. However, by 2 weeks after implantation there was no difference in serum PSA levels among the three groups of animals. All three sets of animals responded similarly to castration with a >85% drop in serum PSA by 1 week after castration. All three sets of animals also showed androgen-

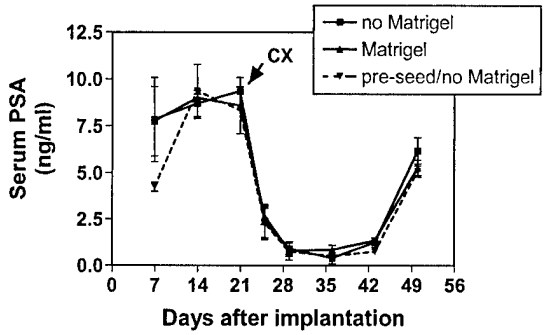

Fig. 3. Matrigel is not required for PSA responses to androgen. Hollow fibers containing LNCaP cells were prepared either with or without the inclusion of Matrigel (50% v/v) and either implanted immediately or pre-seeded 3 days in fibers maintained *in vitro* before implantation into Nude mice. Serum PSA levels were measured in blood samples before and after castration (*CX*) of the animals.

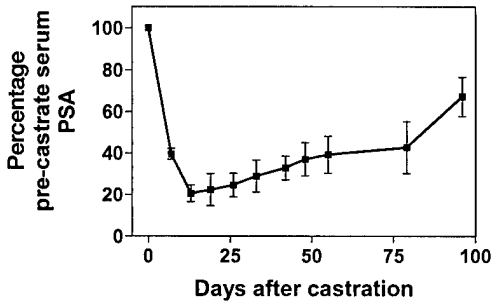

Fig. 4. Androgen-independent increases in serum PSA after castration of Nude mice. One month after s.c. implantation of fibers containing LNCaP cells, the mice were castrated. Precastrate levels of PSA were in the range of 14–41 ng/ml. Data points represent the mean percentage of the precastrate serum PSA (n = 12); *bars,* SE.

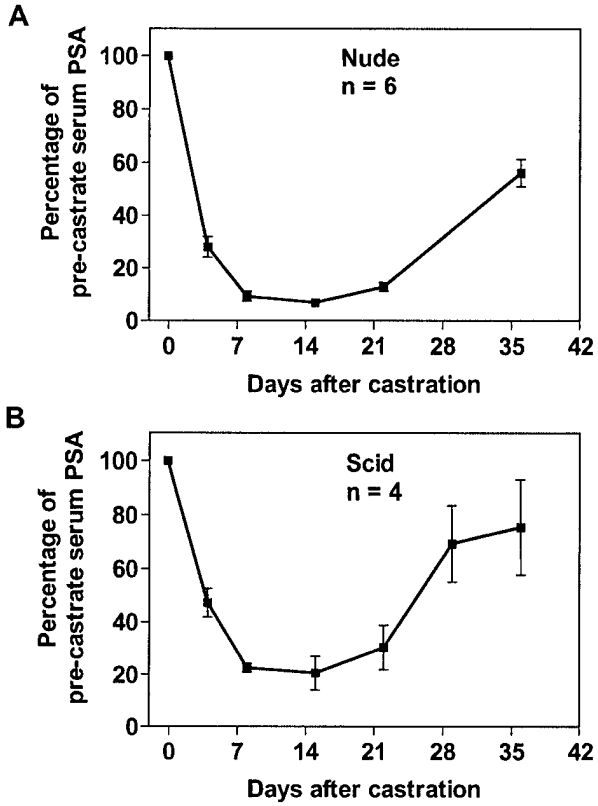

Fig. 5. Differences in serum PSA responses to castration between Nude and SCID mice. Serum PSA levels in castrate Nude (*A*) and SCID (*B*) mice. Fibers contained the same preparation of cells and were implanted s.c. at the same time in both Nude and SCID mice. Precastrate levels of PSA were in the range of 7–10 ng/ml for Nude mice and 8–17 ng/ml for SCID mice. Data points represent the mean percentage of the precastrate serum PSA; *bars,* SE.

independent increases in serum PSA levels over the nadir at 4 weeks after castration. These results suggest that Matrigel is not required for LNCaP cells to grow, respond to androgen, and become androgen independent inside hollow fibers maintained *in vivo* as determined by serum PSA responses. Similar trends were observed in SCID mice (data not shown).

Androgen-independent Increases in Serum PSA after Castration. Androgen deprivation therapy in most patients with prostate cancer results in an 80% drop in serum PSA levels (23). Here we examined the effects of androgen deprivation on serum PSA levels in mice implanted with fibers containing LNCaP cells that were surgically castrated after serum PSA levels were shown to be stable or rising. After castration, serum PSA levels were monitored for an additional 14 weeks. Results from three separate experiments (four Nude mice/experiment) showed that castration resulted in a 80% drop in serum PSA levels by 2 weeks after castration (Fig. 4). Four weeks after castration, serum PSA levels rose again, signifying the emergence of androgen independence. By ~14 weeks after castration, serum PSA levels were increased by 3.5-fold over the PSA nadir.

PSA Responses in Nude Mice Compared with SCID Mice. To determine whether there were differences between Nude and SCID mice in the time to androgen independence and serum PSA response to castration, we performed parallel studies using Nude and SCID mice. All fibers implanted were prepared at the same time; hence, no variables between the experiments could be attributed to differences from the LNCaP cells (*e.g.,* density, passage number, handling, and others). Results in Fig. 5*A* show that castration in Nude mice (n = 6) caused a >90% decrease in serum PSA from precastrate levels. The PSA nadir was reached ~1 week after castration. Five weeks after castration, serum PSA levels were elevated by ~8-fold the nadir value. When comparing serum PSA levels in parallel-treated SCID mice (n = 4), the only difference was the 80% decrease in serum PSA levels from precastrate values by 1 week after castration (Fig. 5*B*). Five weeks after castration, serum PSA levels were 3.6-fold the nadir value, suggesting that the LNCaP cells had become androgen independent. Time to androgen independence was similar between the two sets of animals.

PSA mRNA Levels Correspond to Serum PSA Levels. Using the LNCaP xenograft model of prostate cancer, increases in PSA gene expression in androgen-independent cells is established at the level of transcription (17). Both serum PSA and tumor mRNA levels are down-regulated when testosterone is withdrawn and up-regulated when it is replaced. However, when the tumor becomes androgen-

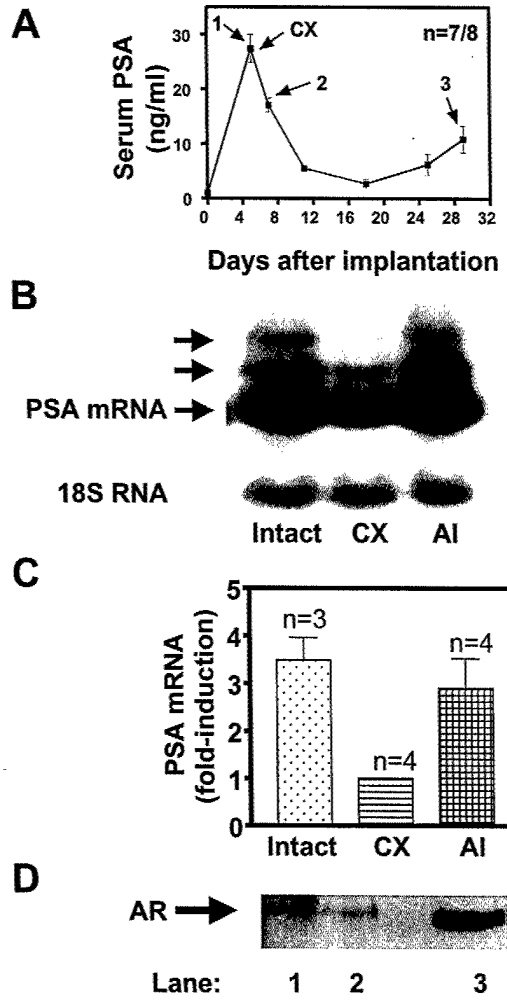

Fig. 6. PSA gene expression in SCID mice that have been implanted s.c. with fibers containing LNCaP cells. *A*, serum PSA levels in blood samples taken over 4 weeks. Animals were castrated (*CX*) at day 5; other *arrows* represent points where cells were harvested and total RNA was isolated for Northern blot analysis. *B*, PSA and 18S RNA levels from cells harvested from a single animal at each of the points indicated in *A* (*Intact*, before CX; *CX*, 4 days after CX; *AI*, 25 days after CX). *Arrows* point to the major 1.6-kb PSA transcript and splice variants (44). *C*, graphic representation of phosphorimaging data of Northern blot analysis of PSA and 18S RNA from cells harvested from a set of animals at each of the time points. Data points represent the mean values of PSA mRNA levels normalized to 18S RNA; *bars*, SE. *n* values are given in the graph over each column. *D*, levels of androgen receptor protein in androgen-independent cells harvested at 25 days after castration (*Lane 1*, whole cell lysate; *Lane 2*, nuclear extract) compared with whole cell levels in LNCaP cells maintained in culture (*Lane 3*).

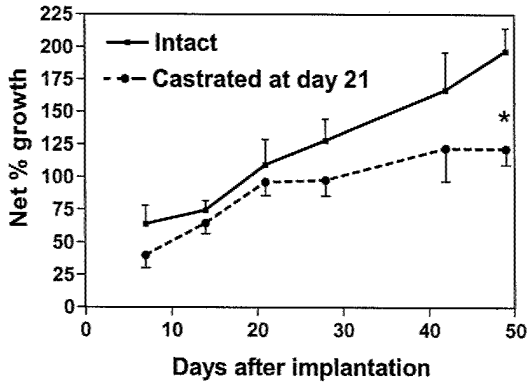

Fig. 7. Proliferation of LNCaP cells grown in fibers and cultivated in Nude mice. Fibers containing LNCaP cells (1×10^7 cells/ml) were implanted into Nude mice and then harvested weekly over a period of 7 weeks for MTT assays. Net percentage of growth in noncastrated animals and animals castrated at 21 days is shown. Data points represent the net percentage of growth defined as: (absorbance on retrieval day − mean absorbance 1 day after implantation) ÷ (mean absorbance 1 day after implantation) multiplied by 100% ($n = 3$); *bars*, SE. *, $P = 0.0127$ (Student's *t* test).

independent, PSA mRNA is constitutively up-regulated despite the continuing absence of testosterone. To test whether this occurs in the LNCaP hollow fiber model, we performed Northern blot analyses on LNCaP cells harvested from fibers in SCID mice at the following time points: (*a*) day 5 when the serum PSA was elevated prior to castration; (*b*) 4 days after castration when the serum PSA was dropping; and (*c*) 24 days after castration when the serum PSA was elevated (Fig. 6*A*). Results from Northern blot analyses of RNA isolated from LNCaP cells harvested at these time points in a single animal are shown in Fig. 6*B*. Northern blot analysis of PSA mRNA levels from three to four different animals, normalized with 18S RNA and bands quantified by phosphorimaging, are shown in Fig. 6*C*. These data indicate that PSA mRNA levels are: (*a*) elevated in the presence of androgen at time point 1 (Intact), which corresponds to elevated serum PSA at this time point; (*b*) decreased at time point 2 (CX), in the absence of androgen, when serum PSA levels are dropping; and (*c*) elevated in the absence of androgen (AI) when serum PSA levels are elevated, signifying androgen independence.

Androgen Receptor Is Expressed in Androgen-independent Cells. A high percentage of clinical specimens of human prostate cancer show expression of androgen receptor both in primary tumors and in hormone-refractory recurrent tumors (24–26). To determine whether androgen-independent LNCaP cells still expressed androgen receptor in this hollow fiber model, whole cell lysates and nuclear extracts were prepared from androgen-independent cells harvested at time point 3 shown in Fig. 6*A*, and Western blots were performed. Androgen receptor was detectable in LNCaP cells in both whole cell lysates (Fig. 6*D*, *Lane 1*) and nuclear extracts (*Lane 2*) harvested after castration when the PSA become elevated again, signifying androgen independence. Thus, androgen receptor was still expressed in androgen-independent LNCaP cells.

Cell Growth. One of the most beneficial aspects of this model is that cell number within fibers can be determined at any point in the experiment. To determine whether LNCaP cells proliferate in this model, we assessed the seeding and harvested cell densities using the MTT assay to calculate the net percentage of growth (15). In the group of intact animals, cells seeded at a density of 1×10^7 cells/ml continued to proliferate over the duration of the experiment at 48 days after implantation of the fibers (Fig. 7). The rate of proliferation was relatively constant during this time, and 196.8 ± 18.8% net growth was calculated for the duration of the

experiment. In the group of animals that was castrated at 21 days after implantation, the initial rate of proliferation in the presence of testosterone was similar to that observed in the intact animals, as would be expected. However, after castration, the rate of proliferation was markedly decreased as indicated by the change in slope after 21 days. A reduced net percentage of growth of 121.5 ± 12.1% was calculated for LNCaP cells maintained in animals castrated at day 21 at the duration of the experiment, as compared with the net percentage of growth for the intact animals.

Response to Intermittent Androgen Suppression. In ~30% of animals, the cells grown in fibers failed to become androgen independent after extended periods in the castrated host. To test whether this was attributable to the loss of cell viability from possibly necrosis or apoptosis, we inserted a testosterone pellet s.c. to determine whether the cells would still respond to androgens after these lengthy periods of cultivation in the animals. Results presented in Fig. 8*A* show that in Nude animals that did not become androgen independent 10 weeks after castration, the implanted LNCaP cells still responded to testosterone, as indicated by the rapid and robust increase in serum PSA to values greater than precastrate levels. Upon removal of the testosterone pellet, serum PSA levels dropped by >90%. Similarly, LNCaP cells maintained in fibers in SCID mice that did not become androgen independent 7 weeks after castration still responded robustly to testosterone as indicated by the 2-fold increase in serum PSA levels over precastrate values (Fig. 8*B*). Animals that did not become androgen independent were next examined to test their response to several cycles of androgen withdrawal and replacement. When the testosterone pellets were inserted, each of the animals responded with a robust increase in serum PSA levels above precastrate values (Fig. 8*C*). When the testosterone pellet was removed, serum PSA returned to nadir levels. Serum PSA response to testosterone could be observed over numerous intervals reflecting the clinical scenario observed in patients treated with intermittent androgen suppression (27).

Tumor Morphology and Histology. Harvested fibers containing cells were fixed in 10% buffered formalin, embedded in paraffin, sectioned (longitudinal and cross-section), and stained with H&E to evaluate the cells cultivated within the fiber. These studies demonstrated that initially the cells grew along the wall of the fiber. In intact animals, the cells tended to stack up, forming layers and scaffold structures resembling a solid tumor with areas that contained necrotic populations (Fig. 9*A*). Cells grown in the presence of Matrigel and maintained *in vivo* in fibers is also shown (Fig. 9*B*). Surprisingly, some fibers removed from castrated animals with elevated levels of serum PSA contained spheroids of cells that had detached from the fiber wall (Fig. 9*C*).

Reculturing Cells. One potential application of this model is to develop new sublines of cells with different requirements for androgen. Presently, there are limited prostate cancer cell lines that are androgen independent and still express androgen receptor (28). To develop such a cell line using the hollow fiber model, androgen-independent cells were harvested from castrated animals with increasing serum PSA and recultured and maintained *in vitro*. While reculturing *in vitro* in RPMI 1640 supplemented with 10% DCC-FBS, many cells did not attach to the plate surface, and clumps of cells floated on the surface of the medium. However, when the cells were able to attach and grow, they resembled neurons, with a small cell body and long dendrite-like outgrowths (Fig. 10).

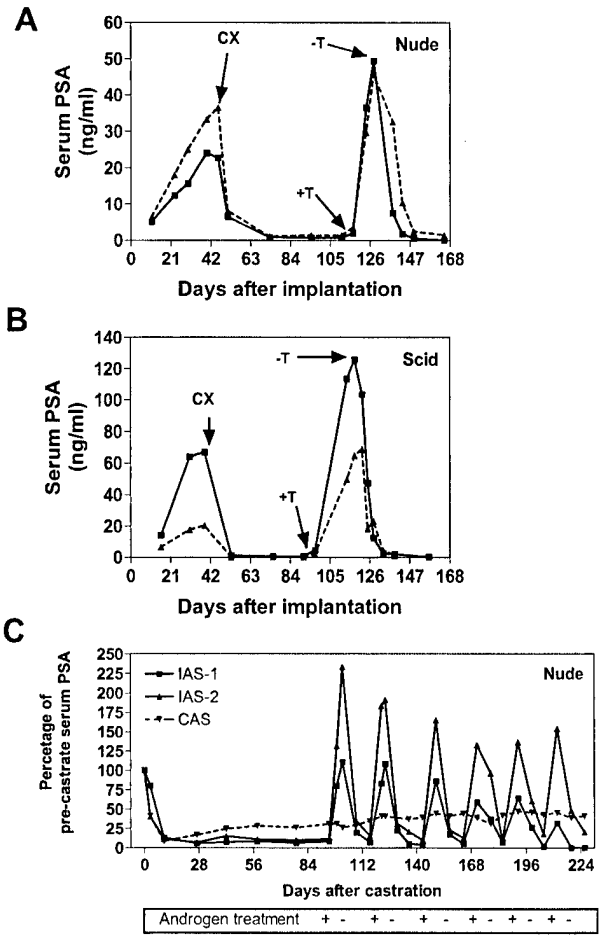

Fig. 8. Changes in serum PSA levels in mice implanted with fibers containing LNCaP cells that did not become androgen independent after castration and their response to testosterone. *A*, two nude mice were castrated 46 days after implantation with fibers containing LNCaP cells and did not become androgen independent by day 115 when testosterone pellets were inserted s.c. These pellets were removed 128 days after castration. *B*, two SCID mice were castrated 39 days after implantation with fibers containing LNCaP cells and did not become androgen independent by day 95 when testosterone pellets were inserted s. c. These pellets were removed 122 days after castration. *C*, two Nude mice (*IAS-1* and *IAS-2*) were castrated 22 days after implantation with fibers containing LNCaP cells and did not become androgen independent by day 95, when testosterone pellets were inserted s.c. and removed at 102 days, inserted at 116 days and removed at 124 days, inserted at 143 days and removed at 150 days, inserted at 164 days and removed at 171 days, inserted at 184 days and removed at 192 days, and inserted at 205 days and removed at 212 days. *IAS*, animals treated with intermittent androgen suppression. *CAS*, serum PSA in a Nude mouse from the same experiment that did become androgen independent and was not implanted with a testosterone pellet (continuous androgen suppression). Precastrate levels of PSA were in the range of 18–23 ng/ml.

Discussion

Development of LNCaP hollow fiber model for the study of progression of prostate cancer to androgen independence is

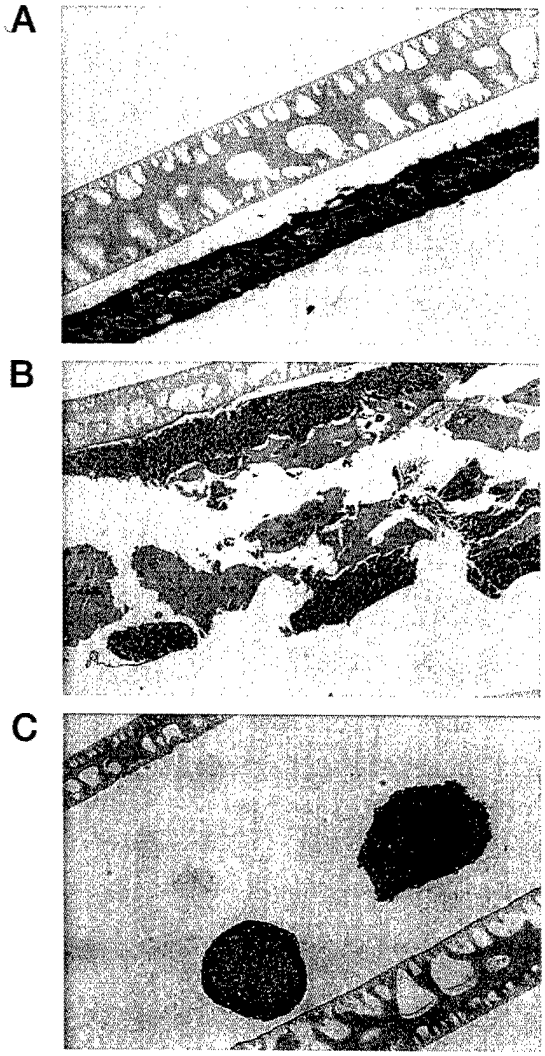

Fig. 9. Histology of LNCaP cells grown inside of hollow fibers and maintained *in vivo*. *A*, LNCaP cells grown inside hollow fibers in intact animals (presence of androgens). *B*, LNCaP cells grown with Matrigel inside hollow fibers in intact animals (presence of androgens). *C*, LNCaP cells grown inside hollow fibers in castrated animals (absent of androgens). Longitudinal sections of fixed tissue stained with H&E.

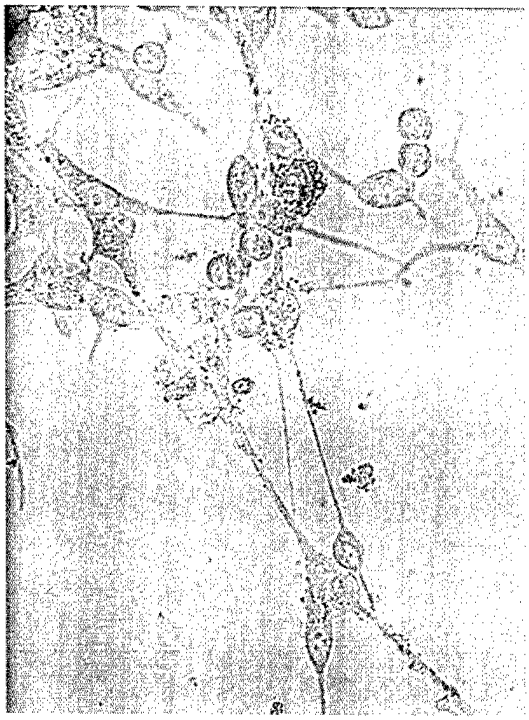

Fig. 10. Androgen-independent cells harvested from castrated animals and recultured in RPMI 1640 supplemented with 10% DCC-FBS.

shown here for the first time. In the course of developing this model, several observations were made. These include: (*a*) Matrigel and direct cell-cell interactions between LNCaP and stroma cells are not required for proliferation or progression of LNCaP cells to androgen independence; and (*b*) androgen-independent increases in serum PSA in castrated animals are uncoupled from proliferation and androgen receptor continues to be expressed in androgen-independent cells.

The LNCaP cell line was established from a prostate metastatic lesion obtained from a patient's lymph node (29). These cells express androgen receptor and markers for prostatic epithelium such as PSA and prostatic alkaline phosphatase. LNCaP cells can be passaged as a monolayer and can form s.c. and intraprostatic tumors in immunocompromised mice (14, 29, 30) that will progress to androgen independence in castrated animals, thereby making this cell line extremely attractive for prostate cancer research. Here we have expanded the applications of this cell line by development of the LNCaP hollow fiber model, which prevents host cell contamination as measured by reverse transcription-PCR for a murine-specific gene using RNA isolated from the fibers (data not shown) and as reported previously (15). At a seeding density of 1×10^7 cells/ml in 16 fibers of 3-cm lengths, for a total of approximately 4.8×10^6 cells/animal, PSA can easily be measured in the blood of SCID and Nude mice. This cell density falls into the range reported previously for other cell lines to achieve optimal cell growth in hollow fibers maintained *in vivo* (15). LNCaP cells seeded at this cell density provided measurable serum PSA levels that increased in intact (noncastrated) animals, decreased by 80% to a nadir after castration, and subsequently increased by 4 weeks after castration. *In vivo* proliferation of LNCaP cells in the fibers continued in the presence of androgens and continued to increase, albeit at a slower rate, in the castrated animals. Data from this fiber model for both serum PSA and proliferation of LNCaP cells are consistent with those data obtained with the LNCaP tumor model (13). In the LNCaP tumor model, the time to androgen-independent progression was shown to be variable, ranging from 14 to >100 days, with a mean of 28 days (31).

Stromal and epithelial interactions are important in the development of the prostate (32, 33). Interactions between these two cells types have also been suggested to play an important role in carcinogenesis and progression (13, 34–37). However, little evidence has been shown as to whether

direct cell-cell interactions are required or whether merely soluble factors are sufficient. One suggestion as to the underlying requirement for Matrigel for the LNCaP xenograft to be successful is that Matrigel provides essential growth factors required for the development of LNCaP tumors (38). However, in the original report by Horoszewicz et al. (29), neither the inclusion of Matrigel nor coinoculation of stromal cells with LNCaP cells were required for tumor take. The LNCaP hollow fiber model that we present here provides evidence that direct stromal-epithelial interactions are not required to achieve androgen-independent increases in PSA. Nor are these direct interactions required for proliferation of epithelial cells in the presence or absence of androgens because MTT results indicate that LNCaP cells still proliferate inside of the fibers regardless of castration. Whether soluble factors from surrounding host cells are required for this proliferation and androgen-independent expression of PSA remains to be determined. There are several lines of evidence that support the role of soluble growth factors from other cells influencing the proliferation of prostate cancer epithelial cells. The first line of evidence can be drawn from the fact that prostate epithelial cells are stimulated to grow *in vitro* by soluble growth factors released from fibroblasts (39). The second line of evidence can be drawn from observations made in the present study that not all of the LNCaP cells maintained in fibers in a set of mice became androgen independent after castration of the host. These differences are most likely attributable to the individual animals because each set of the animals was implanted with fibers prepared at the same time; hence, no variables from the cells were introduced.

In the intact noncastrated animal, serum PSA continued to rise when seeded at the optimal density in fibers (1×10^7 cells/ml) for the duration of the experiment (Fig. 2). Comparison of the rises in serum PSA after implantation (Fig. 2) to the MTT data measuring proliferation of cells (Fig. 7) suggests that the rises in PSA are attributed to proliferating LNCaP cells in the presence of androgens. However, this correlation does not hold true for the castrated animal. In these animals, serum PSA drops by at least 80% within 2 weeks after castration (Figs. 3–6 and 8). This drop in PSA does not correspond to the MTT data generated that suggest that proliferation still occurs, albeit at a slower rate, after castration (Fig. 7*B*). Thus, when serum PSA and mRNA levels are greatly attenuated in castrated hosts, the cell numbers are increasing, thereby uncoupling proliferation from PSA gene expression. This suggestion is consistent with previous observations that LNCaP tumor volume stabilizes but does not decrease after castration, despite the 80% decrease in serum PSA values (14).

Histology studies of LNCaP cells grown *in vivo* inside of fibers showed that these cells form a solid tumor. This is consistent with the reported growth of other cell lines cultivated *in vivo* in hollow fibers (15, 18). In the presence of androgens, LNCaP cells tended to stack up in layers along the fiber wall. At the cell density used for these experiments (300,000 cells/3-cm fiber), at no time was the fiber completely filled with cells to indicate volume constraints. Cells cultivated in fibers for long periods of time in castrated animals tended to form spheroids that no longer grew along the fiber wall. This is the first report to show this phenomenon. Consistent with this was the observation that upon reculturing of androgen-independent cells, many of the cells did not attach to the surface of the plate and formed clumps of cells that floated on the surface of the medium. When androgen-independent cells were recultured, their morphology was altered such that the cells resembled neurons. This change in morphology was similar to that described for LNCaP cells maintained in monolayer and exposed to interleukin 6 (40) and compounds that increase cellular levels of cAMP (41). This change in morphology has also been associated with the differentiation of LNCaP cells into neuroendocrine cells such that markers for neuroendocrine cells, chromogranin A and neuron-specific enolase, become expressed (41). Neuroendocrine cells have been reported to play a role in the progression of prostate cancer to androgen independence (reviewed in Ref. 42). Thus, the observed changes in the morphology of LNCaP cell to resemble neuroendocrine cells in response to castration support the suggestion that androgen ablation may lead to a more aggressive phenotype of prostate cancer (43).

In summary, we have developed the LNCaP hollow fiber model, which provides a reproducible means of obtaining "pure" populations of LNCaP cells during different stages of progression to androgen independence for molecular analysis requiring uncontaminated RNA and protein extracts. However, other possible applications for this model include studies of the transcriptional regulation of genes that are altered during progression to androgen independence and developing new sublines of cells with differing requirements for hormone. Application of this model is not intended to replace xenografts because it cannot reflect the contributions of cell-cell interactions or angiogenesis on tumor biology.

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